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Evaluation of the anti-cardiolipin antibody test: report of an international workshop held 4 April 1986

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SUMMARY

Thirty laboratories from institutions in Britain, France, Italy, The Netherlands, New Zealand, Sweden and the USA participated in a workshop to evaluate the anti-cardiolipin (aCL) test. Participants were asked to measure IgG and IgM aCL in seven samples on each of three separate days. The seven samples were prepared so that IgG and IgM aCL concentrations were known before distribution. Twenty-three of 30 laboratories measuring IgG aCL had significant regression slopes ($P<0{\cdot}001$) when optical absorbance readings or counts per minute were compared with IgG aCL concentration. Twenty-four of 28 laboratories measuring IgM aCL had significant regression slopes ($P<0{\cdot}001$). Coefficient of determination (R^2) ranged from 81·1% to 98·7% for laboratories with valid IgG aCL assays and from 48·0% to 96·7% for valid IgM aCL assays. Valid assays had in common the use of 10% fetal calf or 10% adult bovine serum in PBS. Assays that were not valid had in common the use of PBS, PBS-Tween, or 0·3% gelatin as diluents. All laboratories with valid assays defined samples with high and moderate aCL levels as positive but there was no consensus about low positive samples. This study shows that properly performed ELISA or SRIA assays can be used to provide an accurate, reproducible, and quantitative measure of IgG and IgM aCL concentration in serum samples.

Keywords anti-cardiolipin antibody thrombosis ELISA assay fetal loss

INTRODUCTION

Preliminary studies have suggested that the anti-cardiolipin antibody (aCL) test is important clinically in identifying a group of patients prone to episodes of recurrent thrombosis, fetal loss, and/or thrombocytopenia (Editorial, 1985; Harris *et al.*, 1985a). Since the first published report of a radioimmunoassay method to detect anti-cardiolipin antibodies (Harris *et al.*, 1983), many other centres have reported data using various modifications of this test (Koike *et al.*, 1984; Norberg *et al.*, 1984; Colaco & Male, 1985; Tincani *et al.*, 1985; Lockshin *et al.*, 1985; Loizou *et al.*, 1985; Meyer *et al.*, 1985; Gharavi *et al.*, 1987). Given the potential importance of the test and its relative 'novelty', we believe that it has become necessary to standardize the aCL test before its widespread adoption as a routine laboratory test.

Recent clinical reports have suggested that thrombosis and fetal loss are more frequent in patients with 'high' compared to those with 'low' aCL levels (Lockshin *et al.*, 1985; Harris *et al.*, 1986) and there have been reports that 'lowering' anti-phospholipid (aPL) levels in women with

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these antibodies and fetal loss, using steroid therapy, may result in live births (Lubbe *et al.*, 1984; Branch *et al.*, 1985). If these clinical observations are to be substantiated, it will be necessary, as a minimal requirement, that laboratories will be able to distinguish *correctly* and *reproducibly* between samples with varying aCL levels. It would be helpful, too, to adopt some unit of measurement that would enable exchange of results between laboratories. In addition, for laboratories wishing to set up the aCL test, some method should be available for evaluating which of the published assay methods give valid results.

The aCL standardization workshop was designed to achieve some of the objectives outlined above. There is some evidence to suggest that antibody isotypes may be important clinically (Harris *et al.*, 1986), hence the workshop was designed to evaluate measurement both of IgG and IgM aCL. Seven samples were prepared in such a way that concentrations of IgG and IgM aCL could be estimated in each sample by measurements that were largely independent of the ELISA or SRIA techniques being evaluated. This enabled an objective measure of the accuracy and reproducibility of assay methods used to estimate aCL in serum samples. We were also able to recommend a unit of measurement of aCL assays and to determine which assay methods provided valid results.

METHODS

Preparation of samples. Six of the seven samples were prepared by mixing various proportions of sera from two patients and a normal person. Both patients A and B had the lupus anticoagulant and histories of multiple thromboses compatible with the 'anti-phospholipid syndrome' (Harris *et al.*, 1985a), but patient A had high levels of IgG aCL alone, and patient B had high levels of IgM aCL alone.

Preparations of affinity purified IgG and IgM aCL antibodies from sera of patient A and patient B, respectively, were prepared (Harris *et al.*, 1985b). These affinity-purified preparations were characterized by Ouchterlony and immunoelectrophoresis (IEP) and antibody concentrations determined by the Mancini method. Serial dilutions of the affinity-purified preparations were compared with patient sera from which these preparations were obtained using a modified aCL ELISA technique (Gharavi *et al.*, 1986). On the basis of these results, the concentration of IgG aCL in sample A was estimated to be approximately 320 μg/ml and the concentration of IgM aCL in sample B to be approximately 82 μg/ml. For the purpose of this study, sample A was designated as having an IgG aCL binding activity of 320 GPL units, where 1 GPL unit was taken as the binding activity of 1 μg/ml IgG aCL, affinity-purified from sample A. Sample B was designated as having 82 MPL units, where 1 MPL unit was taken as the binding activity of 1 μg/ml IgM aCL, affinity-purified from sample B.

A total of 25 ml of each of seven test samples were prepared by mixing various proportions of serum samples A, B and N. We were, therefore, able to calculate a GPL and MPL value for each test sample based on the proportion of samples A, B and N in that test sample. For example, if in preparing a test sample, we mixed 10 ml of sample A (320 GPL), 5 ml of sample B (82 MPL), and 10 ml of sample N (zero GPL, zero MPL), the concentration of IgG aCL in the test sample would be $10/25 \times 320$ GPL units and of IgM aCL, $5/25 \times 82$ MPL units. Using this method, we were able to prepare seven test samples whose concentrations together encompassed the full sensitive range of ELISA and SRIA assays for IgG and IgM aCL (Figs 1a, b).

Each participating laboratory was given seven 1 ml bottles, each bottle containing 200 μl of a freeze-dried standard serum. An instruction sheet was provided which described how to prepare the samples for measurement of aCL. Three forms were also provided to enter results of IgG, IgM and IgA (optional) aCL values for each sample on three different days. Laboratories were requested to give a brief summary of their assay method.

Before distribution, three bottles of each of the seven test sera were selected at random and IgG and IgM aCL determined by SRIA and ELISA to ensure that there were no significant differences between bottles containing the same freeze-dried standard samples.

Statistical methods. A plot of logarithmic values of IgG or IgM aCL concentrations encompassed by the seven standard samples against logarithmic value of OD or ct/min gave an

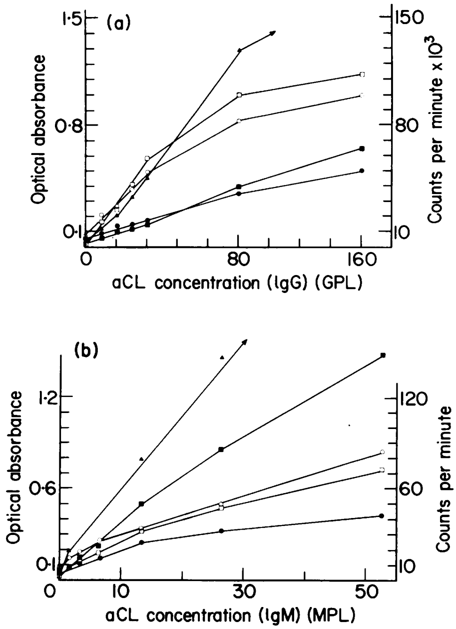

Fig. 1. Relationship between optical absorbance and IgG aCL concentrations (Fig. 1a) or IgM aCL concentrations (Fig. 1b) for five laboratories, two with solid phase radioimmunoassays (▲, Lab. 121; ■, Lab. 12) and three with enzyme-linked immunosorbent assays (●, Lab. 19; ○, Lab. 24; □, Lab. 50).

approximately linear relationship. A linear regression equation was derived for each laboratory as shown below:

$$\mathrm{Log}_e(Y) = a + b.\ \mathrm{Log}_e\,(X)$$

where Y is OD reading or ct/min; X is IgG or IgM aCL concentration; a is the intercept and b is the slope.

Analysis of variances for linear regression was carried out on results of each laboratory to test for significance of: (1) Linear regression, i.e. to test whether the common slope was significantly different from zero. (2) Between-day variation, i.e. to test for parallelism of the slopes for the different days, and shifts in the position of the regression line.

Assay methods were defined as acceptable only if the linear regression was statistically significant at the 0·1% level.

RESULTS

Seven test samples were distributed to each of 39 laboratories. Results were obtained from 30 of the 39 laboratories. All 30 laboratories measured IgG aCL in the seven test sera and 28/30 measured IgM aCL. Twenty of the 30 laboratories measured IgG aCL, and 20/28 laboratories measured IgM aCL in all samples on three different days. Seven laboratories measured IgG aCL and five laboratories measured IgM aCL on two different days. Three laboratories measured IgG aCL and IgM aCL on one day only.

Twenty-six of the 30 laboratories used an ELISA technique, three laboratories used a solid phase radioimmunoassay technique, and one laboratory used a diagnostic kit (Cheshire Diagnostics, Cheshire, UK), based on an ELISA technique.

Regression significance and coefficient of determination. A laboratory was defined as having a

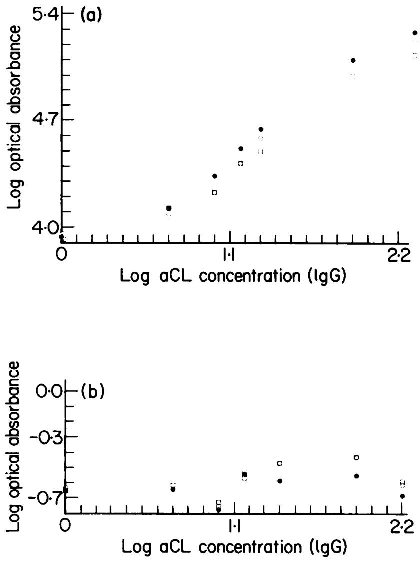

Fig. 2. Example of approximate linear relationship between logarithmic values of optical absorbance and logarithmic values of IgG aCL concentrations encompassed by the seven standard samples for (a) a laboratory with a valid assay and (b) a laboratory with an assay that was not valid. (a) (●) Day 1, (○) day 2, (□) day 3; $R^2 = 98{\cdot}7\%$, F regression $= 1731$ ($P < 0{\cdot}001$), F between day = NS, $\log_e Y = 3{\cdot}047 + 0{\cdot}828 \times \log_e (X)$. (b) (●) Day 1, (○) day 2, (□) day 3; $R^2 = 20{\cdot}6\%$, F regression = NS, F between day = NS, $\log_e Y = -1{\cdot}365 + 0{\cdot}071 \times \log_e (X)$.

valid assay only if the linear regression was statistically significant at the 0·1% level. There were no laboratories where the linear regression was significant between 5% and 0·1% level.

Twenty-three of the 30 participating laboratories measuring IgG aCL and 24 of the 28 laboratories measuring IgM aCL were valid according to the above criteria. All 23 laboratories with a valid test for IgG aCL had coefficients of determination, R^2, ranging from 81·1% to 98·7% (Fig. 2a). The six laboratories with non-valid IgG aCL assays had coefficients of determination, R^2, ranging from 0·03% to 34·0% (Fig. 2b). Coefficients of determination, R^2, for IgM aCL varied from 48·0% to 96·7% for laboratories with valid assays, and, 0·0% to 0·2%, for laboratories with non-valid assays.

All 23 laboratories with significant linear regression values of IgG aCL had slopes greater than 0·20 (Fig. 2a), and all seven laboratories with linear regression values which were not statistically significant had slopes less than 0·10 (Fig. 2b). For IgM aCL, the 24 laboratories with valid tests had slopes greater than 0·2, and all four laboratories with non-valid tests had slopes less than 0·05.

Between-day variations. Only six of the 20 laboratories measuring IgG aCL on more than one day and 4/24 laboratories measuring IgM aCL had significant variations in 'between-day' readings. No laboratory had a significant difference in the slopes between different days. Significant between-day variations need not invalidate an assay method and usually reflects the fact that OD readings may vary from day to day because of slight variations in experimental conditions. Despite these variations, the relationship between OD readings and aCL concentrations remained relatively constant.

Determination of level of positivity. The seven test samples could be ranked according to their IgG aCL (Table 1) or IgM aCL concentrations (Table 2). Sample No. 62, which consisted entirely of serum from a healthy patient, was assumed to have no IgG or IgM aCL.

For IgG aCL, one of the 23 laboratories with valid assays did not indicate which of the seven samples were considered positive. All of the remaining 22 laboratories listed samples 35 (160 GPL), 71 (80 GPL) and 17 (20 GPL) as positive. Twenty of the 22 laboratories considered sample 26 (15 GPL) as positive and one each of the remaining laboratories took this sample as 'doubtful positive'

Table 1. The seven test samples ranked according to IgG aCL concentration and number of laboratories defining each of the seven samples as positive, doubtful positive, or negative

Sample no.	IgG Concentration (GPL units)	No. positive	No. doubtful positive	No. negative
1	160	22	0	0
2	80	22	0	0
3	20	22	0	0
4	15	20	1	1
5	10	15	5	2
6	5	11	4	7
7	0	2	0	20

Table 2. The seven standard samples ranked according to IgM aCL concentration and number of laboratories defining each of the seven samples as positive, doubtful positive or negative

Sample no.	IgM concentration (MPL units)	No. positive	No. doubtful positive	No. negative
1	52	21	0	0
2	26	21	0	0
3	13	20	1	0
4	6·5	18	1	2
5	3·2	14	3	4
6	1·6	9	4	8
7	0	0	0	21

and negative. The remaining results are summarized in Table 1. Of note was the fact that 20/22 laboratories considered sample 62 (0 GPL) as negative.

For IgM aCL, 21 of the 24 laboratories with valid assays indicated which of the seven samples were considered positive. All 21 laboratories listed samples 17 (52 MPL) and 71 (26 MPL) as positive (Table 2). Twenty of the 21 laboratories considered sample 26 (13 MPL) as positive and the remaining laboratory considered the sample as 'doubtful positive'. Other results are summarized in Table 2. All 21 laboratories agreed that sample 62 (0 MPL) was negative.

The differences between laboratories in assigning positivity to samples with low aCL concentrations illustrate the difficulty in determining a mutually agreeable 'cut-off' point and may explain why there may be quite wide variations in reports of percentages of patients 'positive' for the aCL test.

Evaluation of materials and methods used by participating laboratories. There was no single computed measurement that enabled us to determine whether one given assay method was better than the other. Assays best able to measure IgG and IgM aCL concentrations over the range of concentrations encompassed by the seven standard samples had the following features in common: a coefficient of determination, R^2, of 85·0% or greater, and a linear regression equation with a slope greater than 0·35. A summary of the various assay methods that achieved these goals is shown in Table 3.

All assay methods that were not valid had the following features in common: the use of phosphate buffered saline (PBS) alone, PBS/Tween, or 0·3% gelatin/PBS as diluent of serum

Table 3. Summary of assay conditions used by laboratories with valid assays

Major stages of anti-cardiolipin assay	Materials and methods used in assays considered satisfactory	
Plates coated with cardiolipin	Source	usually Sigma.
	Concentration	12 – 100 μg/ml; 25–40 μl/well.
	Solvent evaporation	under nitrogen; under vacuum; 4°C overnight.
Plates blocked	Blocking materials	10% fetal calf serum (FCS)/PBS 10% adult bovine (or ox) serum (ABS)/PBS; 1% bovine serum albumin (BSA)/PBS; 1·5% BSA/0·3% gelatin/PBS.
	Time	1 – 4 h at room temperature.
Patient serum	Dilution	1/50 and below (dilutions less than 1/50, e.g. 1/25 appeared less reliable).
	Diluent	10% FCS; 10% ABS; 0·1% BSA; 0·75% BSA/0·3% gelatin.
	Incubation time	1 – 4 h (overnight with 0·1% BSA in one instance) at room temperature.
Anti-human antibody (enzyme-labelled in ELISA I-labelled in	Material	usually a.p. goat or a.p. rabbit anti-human IgG or anti-human IgM in the same diluent as used for serum. Laboratories using BSA/gelatin for patient serum dilution used 1% BSA as diluent here.
SRIA)	Incubation time	1 h to overnight (SRIA used overnight incubation) at room temperature.
2nd antibody	Some laboratories had an additional step, first using a.p. goat anti-human IgG or IgM in the previous step and then going to a second step using enzyme labelled a.p. sheep or rabbit anti-goat IgG.	

Subsequent procedures are the same for any ELISA or SRIA technique
a.p., affinity purified.

samples, and of anti-human antibody preparations. The source of antigen did not appear to affect results. Two of the assays that did not work either used phospholipid micelles or a mixture of phospholipids to coat microtitre plates, but our experience suggests that these procedures alone should not have caused these assays to fail, and reasons for failure appear to have been the use of gelatin both as diluent of standard sera and of anti-human antibody preparations, and/or incubations of plates at 37°C.

DISCUSSION

The primary finding of this co-operative study was that a variety of ELISA or SRIA methods can provide an accurate and reproducible measure of IgG aCL and IgM aCL, over a concentration range of about 100 GPL units to approximately 5 GPL units for IgG, and from 50 MPL units to approximately 1·6 MPL units for IgM aCL. Although OD or ct/min readings for individual samples may vary from day to day, the essentially linear relationship between logarithm of OD (or ct/min) and logarithm of aCL concentrations does not change significantly over the range of concentrations encompassed by the seven standard samples. Thus, by using four to six standard samples on each assay plate which together cover a wide range of IgG and IgM aCL concentrations, and by assigning some 'unit' of measurement to these standard samples related to aCL concentration, aCL assays can be standardized so that they provide an estimate of aCL concentration in unknown serum samples.

Determination of which of the seven test samples were positive was difficult only at low IgG and IgM concentrations. Instead of defining a 'cut-off' point we suggest that sample results be reported as 'high', 'medium', or 'low' positive. We suggest that an IgG aCL value above 80 GPL units be defined as 'high' positive. The range 15–80 GPL units over which the majority of valid assays were most sensitive, can be defined as 'medium' positive, and levels below 15 GPL units be defined as 'low' positive (Fig. 1a). For the IgM aCL, we suggest that levels above 50 MPL units be defined 'high' positive, those levels between 6·0 and 50 MPL units be defined as 'medium' positive and those with levels less than 6·0 MPL be defined as 'low' positive (Fig. 1b). This method of reporting results, although vague, provides some objective means of exchanging information. The results of this study suggest that all laboratories with valid assays can correctly identify the 'high' positive and 'medium' positive aCL samples and significant differences only arise in reporting 'low' positive results.

Although unable to determine with accuracy why some assays seemed to provide 'better results' than others, this study was able to pin-point certain common problems to assays that were not valid. The use of PBS alone, PBS-Tween, or 0·3% gelatin as diluent of both serum samples and anti-human antibody, as well as warming plates to 37°C, were features only of assays that were not valid. Most laboratories with valid assays used 10% fetal calf serum or 10% adult bovine serum for blocking plates, as diluent of serum samples and as diluent of enzyme-labelled or ^{125}I-labelled antihuman antibody. Other variables such as incubation time and choice of anti-human antibody may also have affected the quality of results.

If, as some investigators currently believe (Harris *et al.*, 1985a), anti-cardiolipin antibodies include sub-populations of antiphospholipid antibodies with lupus anticoagulant activity, then a standardized aCL test will be a better instrument for detecting and measuring anti-phospholipid antibodies than the lupus anticoagulant (LA) test. Significant variations in the tests used to detect the lupus anticoagulant make standardization and reliable quantification of results difficult (Green *et al.*, 1983).

We suggest that the method outlined in this report for evaluation of the aCL test can, in theory, be applied to evaluation and standardization procedures for other auto-antibody tests which utilize ELISA or SRIA techniques.

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